REMARKS/ARGUMENTS

Claims 1-4, 7-11, 14-17 and 27-31 are pending in the application. Claims 3, 5-6, 10, 12-13 and 18-26 have been canceled without prejudice. Claims 1, 8, 16 and 17 have been amended. Reconsideration of the rejection and allowance of claims 1-2, 4, 7-9, 11, 14-17 and 27-31 are respectfully requested.

The Amendment

In order to expedite prosecution of the application and advance the case toward allowance, the claims have been amended. Claims 1, 8 and 17 have been amended to specify that the first filter has a pore size of between about 0.3 and about 1.5 μm. Support for this amendment can be found, for example, on page 7, paragraph 028. Claim 16 has been amended to specify that the preparation is substantially free of contaminating proteins. Support for this amendment can be found, for example, on page 4, paragraph 013. No new matter has been added by this amendment.

Telephonic Interview

The Applicants gratefully acknowledge that the Examiner granted a telephonic interview on March 18, 2005 and has indicated in the Office Action that the unexpected results would be considered further.

35 U.S.C. §103

Claims 1-4, 7-11, 14-17 and 27-31 remain rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Dubensky Jr. *et al.* (USPN 5,789,245, herein "Dubensky") in view of Yu *et al.* (Vaccine (1997) 15(12/13):1396-1404, herein "Yu"), both of record, and further in view of Harley *et al.* (*Clin. Micro. Reviews*, 2001, 14(4):909-932, herein "Harley").

The rejection is respectfully traversed to the extent that the rejection applies to the claims as amended.

Surprising Results

It is known in the art that a low titer of pure virus preparation is a major hurdle to large scale application. The invention improves both, titer and purity of virus product as discussed below. As such, one major advantage of the invention is that the Applicants have developed a method that achieves a very pure virus intermediate through filtering. Notably, the reduction of any residual protein and nucleic acid contamination is important in order to produce a pure virus product that is further applicable to large scale application. For example, the reduction of DNA contamination is a critical step in vaccine production (see FDA publication; *Letter to Sponsors Using Vero Cells as a Cell Substrate for Investigational Vaccine*; 2001; copy enclosed). Particularly, residual DNA (*e.g.*, Vero cell DNA) is of continued concern with respect to viral vaccines according to the Center for Biologics Evaluation and Research (CBER) (see FDA publication, page 1). The Applicants have addressed this concern and have designed a method that achieves a virus preparation which has *less than about 10 pg cellular nucleic acid /µg virus antigen*. The importance of removal of residual DNA from biological products is further emphasized in Smith *et al.* (see *Quantitation of Residual DNA in Biological Products: New Regulatory Concerns and New Methodologies*; Animal Cell Technology: Developments, Processes and Products (1992); Editors R.E. Spier *et al.*; Butterworth-Heinemann; pages 696-698; copy of relevant pages attached). The potential problems associated with such residual DNA include malignant transformation of cells by activated oncogenes, uptake and subsequent expression of viral genomes in cells, and alteration of gene expression by insertion of gene sequences into sensitive control regions of genes (see page 696, second paragraph). The article indicates that although the majority of DNA fragments in residual DNA are too small to harbor complete open reading frames, larger fragments of DNA that are capable of encoding functional proteins are present, and the size distribution of fragments of DNA that are present in the final product will vary with the steps involved in the purification process (see page 697, third paragraph).

Another major advantage of the invention is that the pure virus intermediate of the present invention is *not* substantially reduced during filtration. The Examiner must appreciate that this was unexpected since any filtering process commonly leads to substantial loss of virus

product. The Applicants use a combination of filters that effectively purifies the product without resulting in substantial loss of the intermediate. As such, the Applicants have *surprisingly* found that the enveloped virus passes their filtering system without reduction of virus titer. To that effect, the specification states the following on page 6, paragraph 025:

> It has been **surprisingly** found by the present invention that by filtering the cell culture supernatant derived from cells infected with enveloped viruses (*e.g.*, the Ross River virus), the enveloped virus passes the filter system **without reduction of virus titer,** while cellular contaminants, like proteins and nucleic acid are efficiently removed. The method of the invention provides purification of a high titer virus preparation by filtration, wherein this method is **easily applicable for large-scale purification** and efficiently removes most of the protein derived from the host cells as well as of cellular nucleic acid. The method of the invention therefore provides a process of purifying virus antigen by filtering without remarkable loss of virus titer and virus antigen. [Emphasis added in bold.]

MPEP §2144.08 states that rebuttal evidence may include evidence that the claimed invention yields unexpectedly improved properties or properties not present in the prior art.[1] The Examiner will appreciate that the instant invention achieves *a very pure virus intermediate* through filtering *without* any substantial reduction in virus titer. This is exemplified in Table 1 on page 14, wherein the virus titer ($TCID_{50}$/ml) of the harvest (8.0; 7.6 after separation) was hardly reduced after filtration (7.2). Since the virus titer is shown as $TCID_{50}$/ml, the numbers in the table refer to the following:

8.0 = 1.0×10^8 harvest

7.2 = 1.58×10^7 filtered (= 15.8% $TCID_{50}$ yield)

7.6 = 3.98×10^7 separated

7.2/7.4 = ~2.00×10^7 filtered/Benzonase treated (50% $TCID_{50}$ yield)

Since the Applicants have achieved such a pure virus intermediate (about 97% pure), the final purification method can be selected from any number of methods (*e.g.*, sucrose gradient purification, *etc.*) since residual DNA contamination has already been substantially reduced. In addition, protein contamination in the intermediate product is also substantially reduced through filtering. The Applicants discuss on page 4 of the application (see paragraph

[1] See MPEP §2144.08 (II) (B)

013) that the contaminating proteins and nucleic acids are below the detection limit of the state of the art detection method (*i.e.*, Westernblot analysis and densitometric determination can be used to detect residual proteins while PCR can be used to detect residual DNA).

In comparison, the art uses several steps and/or procedures to achieve a similarly or less pure virus with the added disadvantage that the greater number of steps reduces virus titer and antigen yield. Specifically, the art does not teach a filtering method that achieves a very pure virus intermediate without any substantial reduction in virus titer. As stated on page 6, paragraphs 023 and 024, various methods known in the art are used to remove contaminating products and efficient purification methods often comprise several steps and combinations of methods. Filtration is used in the art to purify biological material, whereby viruses, particularly enveloped viruses, remain in the retenate, and the virus titer in the filtrate is reduced. The Applicants have solved this problem by developing a system that achieves a pure virus preparation without substantial loss of virus titer.

High Purity

The Office Action indicates that the specification defines "purified Ross River Virus antigen" as having greater than about 97% purity as determined by SDS-PAGE and Western blot analysis with anticellular protein specific antibodies and quantification of residual cellular nucleic acid; and that the Applicants point to the viral preparation of greater than about 97% purity following just two filtering steps as proof of surprising results. The Office Action then indicates that the Applicants have not demonstrated that Dubensky's method does not result in an equally pure product. Herein, the Examiner indicates that, although Dubensky describes his product from the filteration steps as "crude", it does not follow that the product was not greater than about 97% pure. The Office Action asserts that it would have been obvious to use a filter pore size of less than 0.65 microns given that the diameter of an alphavirus is known and thus, the determination of the particular filter pore size ranges employed is within the skill of the ordinary worker and a part of the process of normal optimization.

The Office Action is correct in indicating that the purified Ross River Virus antigen is defined as greater than about 97% pure. However, the Examiner may not appreciate

that the Applicants have designed a method that achieves an *intermediate virus product* of about 97% purity. As indicated previously, such a highly purified intermediate product assures a final product of even greater purity (*i.e.*, in addition to no substantial loss in virus titer). This is further explained in the specification on page 7, paragraph 028, which states the following:

> By filtering during virus/virus antigen purification, **substantially all cellular protein contamination is removed.** The cellular **contaminating nucleic acid is also efficiently removed by a factor of at least 35,** and an ***intermediate pure preparation*** having a purity of at least about 97% compared to the starting virus harvest is obtained by this purification step. [Emphasis added in bold.]

Thus, it is clear from the specification that about 97% pure means that the filtering removes nucleic acids by at least a factor of 35 and further removes substantially all cellular proteins. The Examiner will surely appreciate that a 97% pure virus intermediate is a very pure virus preparation considering the current standard in the art which usually achieves virus intermediates of no more than *crude* quality at best (see Dubensky).

> The burden of establishing a *prima facie* case of obviousness falls upon the Examiner. Therefore, the evidence upon which the Examiner relies must clearly indicate that a worker of routine skill in the art would view the claimed invention as being obvious, as meant by 35 U.S.C. §103.[2] [Emphasis added.]

The Office Action speculates that, although Dubensky describes his product from the filteration steps as "crude", it does not follow that the product was not greater than about 97% pure. Yet, the Examiner has provided no evidence why a skilled artisan would ever consider a "crude" virus intermediate as 97% pure. Respectfully, the art understands a "crude" virus intermediate to be a raw or unrefined product that is likely contaminated with substantial amounts of residual protein and DNA. Thus, a "crude" virus intermediate as it appears in Dubensky would never be interpreted as 97% pure by any skilled artisan.

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[2] *Ex parte Wolters and Kuypers*, 214 U.S.P.Q. 735 (PTO Bd. App. 1979).

It is also noteworthy, that Dubensky focus on the use of *recombinant viruses as vectors* (see column 1, Technical Field) and *recombinant alphavirus particles* (see column 120) while the Applicants use *inactivated virus as vaccine*. Dubensky teach the preparation of packaged recombinant alphavirus particles in column 120 (Example 10), wherein they state that "the media exiting the bioreactor is collected and passed initially through a 0.8 micron filter, then through a 0.65 micron filter **to clarify** the crude recombinant alphavirus particles" (see column 120, lines 11-14). In comparison, the Applicants use separation (a kind of centrifugation) as the clarification step (see Table 1 on page 14) and filtration to remove DNA and protein. Finally, Dubensky state that DNase is added to digest exogenous DNA (see column 120, lines 16-18) and cross flow filtration is used (diafiltrate is loaded onto a Sephadex-S-500 gel column) to concentrate the virus (see column 120, lines 18-21). However, there is no discussion in Dubensky about the importance of removing residual DNA from the virus preparation nor is any data provided, nor does Dubensky provide any information on the purity of his final product (see column 120, Example 10).

The Office Action then asserts that it would have been obvious to use a filter pore size of less than 0.65 microns given that the diameter of an alphavirus is known and thus, the determination of the particular filter pore size ranges employed is within the skill of the ordinary worker and a part of the process of normal optimization. Herein, the Examiner appears to assume that a small change in a filter range must be interpreted as an obvious change. Yet, there is no such correlation. Even a small change can be a basis for patentability. <u>The Applicants have designed a system with two filter ranges that effectively *purifies* a virus product *without* substantial loss of virus titer</u>. Alpha virus particles are about 400 Å in diameter which amounts to about 0.04 μm. So far, the Examiner has provided no evidence why a second filter of a pore size of between 0.1 μm and 0.5 μm would be obvious in light of Dubensky and/or the alpha virus particle size of 0.04 μm. <u>Dubensky only achieved a crude virus intermediate by using a 0.65 micron filter and he provides no suggestion that a reduced filter size would provide a purer product without substantial loss of virus titer</u>. There is no motivation to combine Dubensky and/or Yu and/or Harley because neither their individual teachings nor their combined teachings would suggest that a filter size in the range of between 0.1 μm and 0.5 μm would lead to a purer

virus product without loss of antigen yield.

The Office further requests clarification with respect to Table 1. The Applicants are requested to explain how the purity went from 97% to just 98% after the benzonase/gradient steps when the amount of VERO cell DNA decreased from 14,000 to [1] 7000 pg/ml. As the Examiner will appreciate, 7000 to 14,000 picograms of DNA are very small amounts (*i.e.*, 7.0 x 10^{-14} to 14.0 x 10^{-14} g of DNA). Such a small amount of DNA is not expected to affect the final purity of the virus preparation by more than 1 percent if at all. As explained above, "about 97% pure" means that nucleic acids are removed by at least a factor of 35 and cellular protein contamination is substantially removed as well. In fact, the Examiner must appreciate that the small difference between the 97% pure intermediate virus preparation and 98% final virus preparation only emphasizes that the Applicants have indeed achieved a very pure virus intermediate.

Lastly, the Applicants point out the efficiency of the DNA filtration step for the convenience of the Examiner. Table 1 on page 14 of the specification shows the following information in the last column of the table:

DNA reduction:		**residual DNA**
Harvest	6,300pg/µg	100%
Separator	4,200	33%
Filtration	**175**	**3%**
Benzonase	82	1%
Sucrose gradient	5.5	<< 1%

As the Examiner can see, filtration reduced residual DNA in the virus preparation to as low as 3 percent.

In light of the amendments and arguments presented above, it is respectfully requested that the rejection of claims 1-2, 4, 7-9, 11, 14-17 and 27-31 under 35 U.S.C. §103(a) be withdrawn.

Appl. No. 10/006,671 **PATENT**
Amdt. dated July 7, 2005
Reply to Office Action of April 7, 2005

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-273-4703.

Respectfully submitted,

Brigitte A. Hajos
Reg. No. 50,971

Attachments (2)

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Letter to Sponsors Using Vero Cells as a Cell Substrate for Investigational Vaccines

Department of Health and Human Services
Public Health Service
Food and Drug Administration
1401 Rockville Pike
Rockville, MD 20852-1448

Division of Vaccines and
Related Products Applications
Telephone: (301) 827-3070

March 12, 2001

Dear:

The Center for Biologics Evaluation and Research (CBER) is issuing this letter to inform manufacturers (the following interim recommendations pertaining to viral vaccine products that are produced in Vero cel and investigated for human use. These recommendations are based on extensive internal discussions, consultation with outside experts, and comments received from the Vaccines and Related Biological Products Advisory Committee (VRBPAC) during the meeting held on May 12, 2000. In general, CBER currently views Vero cells as an acceptable cell substrate for viral vaccines, but has residual concerns sponsors should attempt to address.

CBER recommends that all products derived from Vero cells be free of residual intact Vero cells. If your manufacturing process does not include a validated filtration step or other validated procedure to clear residual intact Vero cells from the product, please incorporate such a procedure into your manufacturing process and submit the appropriate changes to your IND or MF.

Internal discussions and comments from the VRBPAC suggest the need for continued concern about the level of residual Vero cell DNA in products manufactured in these cells. Although the World Health Organization (WHO) currently accepts a limit of residual DNA from continuous cell lines of 10ng per dos for these products when administered parenterally, CBER wishes to continue considering the level of ris posed by residual Vero cell DNA on a case-by-case basis for viral vaccines. Consideration will also be g to the method of vaccine administration, e.g., parenteral, mucosal, or other route. Based on this concern CBER recommends that you:

a. Measure the amount and size distribution of residual cellular DNA in your final product if you have done so already. Please submit these results to your IND or MF and describe them in terms of the amount of residual cellular DNA per human dose of final formulated vaccine.

b. Consider various methods (e.g., DNAse treatment) by which the amount and size of residual cellu DNA might be further reduced. Please comment on what you have done or intend to do to consid the introduction of additional DNA reducing methods into your process, as well as the potential im of such changes on the performance (e.g., immunogenicity) of the product.

Internal discussions and preliminary comments of the VRBPAC also suggest the need for tumorigenicity testing of each manufacturer's Vero master cell bank and the end-of-production-passage-level-cells (EO derived from this cell bank. The term "EOPC" is meant to include cells at the end of a production run, as as cells cultured from the master or working cell bank to a population doubling level comparable to or beyond cells at the end of production. EOPC should preferably be described in terms of population doublings from your Vero master cell bank. The preferred model for this test is the immunosuppressed newborn Wistar rat, which should be followed for a period of at least five months. Alternative tumorigenic models may also be appropriate in certain circumstances and their use should be discussed with CBER. any evidence of tumorigenic potential is demonstrated in these tests, or if the results are inconclusive, th need for additional tumorigenicity testing with cell lysates and/or DNA will also need to be discussed wit CBER.

Please submit your responses to your IND(s) or MF(s) within six months from the date of issuance of thi letter. Please direct any questions in the interim to Dr. Rebecca Sheets at the telephone number above.

Sincerely yours,

— *signature* —

Karen Midthun, M.D.
Director
Office of Vaccines Research and Review
Center for Biologics Evaluation and Research

Updated May 29, 2002

ANIMAL CELL TECHNOLOGY: DEVELOPMENTS, PROCESSES & PRODUCTS

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QUANTITATION OF RESIDUAL DNA IN BIOLOGICAL PRODUCTS: NEW REGULATORY CONCERNS AND NEW METHODOLOGIES.

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ABSTRACT

The importance of residual DNA testing has been reinforced by two recent observations. First, the application of naked oncogenic DNA to mouse skin resulted in neoplastic transformation, second, intravenous injection of molecularly cloned proviral DNA of a simian immunodeficiency virus resulted in active viral infection. We have developed standardised procedures under GLP guidelines for the detection of residual DNA in biologicals which permit quantitation of contaminants to below 10pg per dose. The testing of samples in duplicate with the inclusion of two or more samples spiked with different levels of exogenous DNA is recommended. We have performed validation experiments which compare our hybridisation based assay with the biosensor-based Threshold™ system developed by the Molecular Devices Corporation. While the level of sensitivity of both assays is less than 10pg DNA, there are certain factors which should be considered in the selection of the assay, including the amount of protein to be evaluated, host species, and the availability of species-specific probes.

INTRODUCTION

The measurement of residual DNA in biological products is part of routine safety testing protocols. Potential problems associated with such DNA include malignant transformation of cells by activated oncogenes; uptake and subsequent expression of viral genomes in cells, and alteration of gene expression by insertion of sequences into sensitive control regions of genes. Risk is assessed on the quantity of DNA present and an arbitrary value of 10pg of residual DNA per therapeutic dose has been set as an acceptable level by regulatory authorities in Europe and the United States of America[1]. As well as amount, the risks are related to the size of the contaminating DNA.

While the alteration of gene expression by insertion of DNA into control regions is well documented, new evidence is accumulating that the risks from the first two events are more than theoretical. Naked plasmid DNA encoding the activated T24 *H-ras* was capable of transforming mouse endothelial cells *in vivo* after direct application of the DNA to scarified mouse skin[2]. Injection of molecularly cloned simian immunodeficiency virus (SIV_{mac}) proviral DNA into susceptible monkeys led to an active infection of three out of four animals[3]. In both cases the amounts of DNA were at least ten-thousand fold greater than that found in purified product but they emphasise the potential risks associated with DNA

RESULTS AND DISCUSSION

DISTRIBUTION OF FRAGMENT SIZE

Purified residual DNA from a typical bulk harvest of final product from murine cells was examined by agarose gel-electrophoresis (Fig. 1). The majority of the DNA fragments were below 200 base pairs (bp) in size, distributed in bands of approximately 200bp, 120bp and 60bp, reflecting size selection during the initial purification process (Fig. 1A, B). In comparison, murine genomic DNA

digested to completion with Alu I, a frequent cutting restriction endonuclease, gave a visible smear of fragments ranging down from 2kbp on electrophoresis (Fig. 1A, c).

In order to detect the full size range of DNA fragments not visible by ethidium bromide staining, the DNA was transferred to a charged nylon membrane by capillary blotting and hybridised with ^{32}P-labelled murine DNA. Figure 1B shows the resulting autoradiogram obtained. Fragments over 6kbp were readily detected in the lanes containing the DNAs (Fig. 1B, c,d). The pattern of bands seen in the restriction endonuclease digested DNA was the result of hybridisation to banded repetitive sequences, the major radio-labelled component of the murine DNA probe.

It is evident from these data that although the majority of DNA fragments in residual DNA are too small to harbour complete open reading frames, larger fragments, capable of encoding functional proteins, are present. The size distribution of fragments of DNA present in final product will vary with the steps involved in the purification process.

SIZE PROFILE OF RESIDUAL DNA

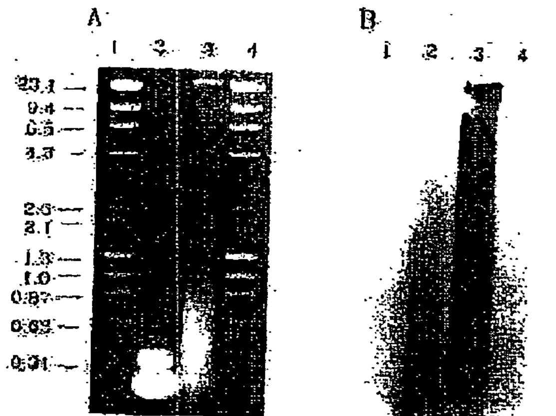

Figure 1. Electrophoresis of residual DNA. A, ethidium bromide stained. B, hybridized with murine probe.

DETECTION SYSTEMS

There are two main methods in current use for the quantitation of residual DNA in final products. First, by hybridisation where the DNA is purified from the test article, usually a protein, bound to a membrane and hybridised with an appropriate radioactively labelled probe[7]. The DNA level is evaluated by comparison of the test article signal with that of the controls from the autoradiogram. The controls should include an extraction of the test article or test article solute spiked with a known amount of DNA to allow an assessment of the efficiency of extraction. The extraction efficiency of DNAs from a test article is affected by a number of factors including: protein concentration, buffer composition (phosphate, EDTA, salts), the volume of the sample to be extracted and the size of the DNA fragments.

The Second method, the *Molecular Devices* Threshold™ system is a potentially more speedy, less labour intensive technique. The system uses two DNA binding proteins with high affinity for DNA but low sequence specificity. One protein is conjugated to an enzyme for signal generation and the other to a hapten for capture of DNA on a membrane. Quantitation is done by measuring enzyme activity through changes in surface potential on a silicon sensor.

Detailed results showing the validation and comparison of both methods will be presented elsewhere (Per and Sito). Briefly, recovery of DNA spiked into murine Mab, after pre-treated with proteinase K/SDS was high. The system also gave equivalent results to hybridization with respect to sensitivity and reproducibility. However, a comparison of the detection efficiency of the kit

control calf thymus DNA to murine, CHO and plasmid DNA revealed that although the Threshold assay was able to detect DNA from other species there was significant variation in the evaluation of DNA content of a series of control dilutions (Table 1).

TABLE 1 Comparison of detection of DNA from different species.

Results: pg detected for 100, 50, 25, 12.5, 6.3, and 3.1 pg DNA tested

Assay 1

	Kit Calf Thymus	Mouse	CHO	Plasmid	Yeast tRNA
100	100.4	172.3	128.9	45.6	0.4
50 (#)	50	72.5	61.2	21.4	0.5
25	34.4	34.0	27.5	9.9	1.0
12.5	11.9	16.4	13.6	4.2	0.2
6.3	6.7	8.1	7.6	2.7	0.8
3.1	4.7	4.8	3.9	1.0	0

CONCLUSIONS

Before using the Threshold device careful validation and standardization for a particular situation are required. In particular the use of calf thymus DNA as standards would be misleading in the measurement of DNA in a test article and a validation of each species' DNA should be performed before measurements are made.

FUTURE

The use of semi-automated devices such as Threshold are valuable in routine testing where multiple identical samples are to be assessed once validated for a particular situation.

Testing of residual DNA for specific sequences is possible using polymerase chain reaction (PCR) assays, a useful safeguard where DNA of a known hazard, e.g. a virus, is likely to be present in a final product. Primers can be chosen such that they will only amplify sequences encoding a complete open reading frames and not small sheared fragments.

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